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Gene expression of vitamin D hydroxylase and megalin in the remnant kidney of nephrectomized rats

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Background. Regulation of vitamin D hydroxylase genes in the early stage of chronic renal failure is not fully understood. Using nephrectomized rats, we examined changes in mRNA levels of CYP27B1 (25-hydroxyvitamin D_3-1α-hydroxylase), CYP24 (25-hydroxyvitamin D_3-24-hydroxylase), and vitamin D receptor in relation to megalin, recently found to participate in renal vitamin D metabolism.

Methods. A rat model of moderate renal failure was induced by 3/4 nephrectomy. Plasma parameters, including vitamin D metabolite concentrations, were measured at weeks 2, 4 and 8, and poly(A)$^+$ RNA extracted from the remnant kidneys was subjected to Northern blot hybridization.

Results. Plasma creatinine concentration at week 2 was 0.40 ± 0.02 mg/dL in the sham-operated and 0.93 ± 0.15 mg/dL in the nephrectomized rats, and both values remained constant up to week 8. Plasma concentrations of 25(OH)D_3, 1α,25$(OH)_2D_3$, and 24,25$(OH)_2D_3$ were unchanged between nephrectomized and sham-operated rats at week 8. Intact parathyroid hormone (PTH) increased at week 8 in nephrectomized rats. CYP27B1 mRNA in nephrectomized rats did not vary at week 2, but increased approximately two- and four-fold at weeks 4 and 8, respectively, compared to the sham-operated rats. CYP24 and megalin mRNAs, on the other hand, began to decline as early as at week 2 in nephrectomized rats and kept decreasing throughout the experiment. The expression of vitamin D receptor was modestly but significantly decreased only at week 8.

Conclusion. Coordinated and reciprocal alterations of the increase in CYP27B1 mRNA and the decrease in CYP24 mRNA may play a pivotal role in maintaining the plasma level of 1α,25$(OH)_2D_3$ in the face of reduced nephron mass and/or megalin expression.

Key words: renal failure, secondary hyperparathyroidism, vitamin D metabolites, vitamin D receptor, messenger RNA.

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The most critical step in the regulation of vitamin D metabolism is the renal conversion of 25-hydroxyvitamin D_3 [25(OH)D_3] to 1α,25-dihydroxyvitamin D_3 [1α,25$(OH)_2D_3$] catalyzed by mitochondrial 25(OH)D_3-1α-hydroxylase (CYP27B1). Another important enzyme in this process is 1α,25$(OH)_2D_3$-24-hydroxylase (CYP24), which breaks down 1α,25$(OH)_2D_3$ and uses the 25(OH)D_3 as a substrate to produce 24,25-dihydroxyvitamin D_3 [24,25$(OH)_2D_3$]. Thus, a balance between CYP27B1 and CYP24 may be one of the major determinants in maintaining the plasma level of 1α,25$(OH)_2D_3$. In addition, both enzymes are regulated by several hormones and ions.

It is believed that a decrease in 1α,25$(OH)_2D_3$ synthesis plays a key role in generating secondary hyperparathyroidism in the setting of chronic renal failure [1]. The mechanism of the decrease in 1α,25$(OH)_2D_3$ was thought to be simply due to the diminished renal mass [2] and thus to the reduced enzyme activity of CYP27B1 localized in the proximal tubules. However, the plasma concentration of 1α,25$(OH)_2D_3$ remains within normal range in chronic renal failure with a glomerular filtration rate (GFR) above 30 to 40 mL/min [3, 4]. Therefore, the mechanism of the normal plasma concentration of 1α,25$(OH)_2D_3$ in this moderate renal failure is probably due to a decrease in degradation of 1α,25$(OH)_2D_3$ [5]. Nonetheless, it has not been clearly shown whether the enzymatic activity is reduced or the gene expression itself is reduced.

Recent evidence has suggested that megalin may be a player involved in renal vitamin D metabolism because megalin knockout mice were unable to retrieve 25(OH)D_3 from the glomerular filtrate, resulting in vitamin D deficiency [6]. Megalin, which has long been called gp330, was originally ascribed as a causative antigen for inducing experimental membranous nephropathy in rats [7, 8]. The protein exists at the intermicrovillar domains of the apical membrane of the proximal tubules. Later, megalin was found to be a multifunctional clearance receptor by means of endocytosis of the many proteins that filter

through the glomeruli [9], including vitamin D binding protein [6].

Therefore, it may be possible that megalin plays some role in vitamin D metabolism in chronic renal failure and in secondary hyperparathyroidism as well. To explore the molecular events of vitamin D hydroxylase gene expression occurring in the early stage of chronic renal failure, the mRNA levels of CYP27B1 and CYP24 in the remaining kidney of nephrectomized rats were measured as a function of time with particular reference to megalin expression.

METHODS

Chemicals

All chemicals were of highly purified analytical grade. [α-^{32}P] deoxycytidine triphosphate (dCTP) was purchased from DuPont/New England Nuclear (Boston, MA, USA), and the random primed DNA labeling kit was obtained from TaKaRa Biomedicals (Shiga, Japan).

Animals

Adult male Sprague-Dawley rats (250 to 280 g) (Japan Clea, Tokyo, Japan) were used. The animals were maintained with a standard commercial diet containing 1.09% calcium and 0.93% phosphorus (Japan Clea) and housed at an ambient temperature of 22°C with an alternating 12-hour light and 12-hour dark cycle. After a 3-day adaptation period, the animals were randomly allotted to sham-operation or 3/4 nephrectomy groups. Three-fourths nephrectomy was done in one step by performing right nephrectomy and a resection of both poles of the left kidney yielding half the weight, as reported previously [10]. One day before weeks 2, 4, and 8 following nephrectomy and sham-operation, the rats were individually housed in a metabolic cage and sacrificed the following day. Blood samples were drawn by puncture of the abdominal aorta and the remnant kidney was excised and subjected to total RNA extraction.

The animal studies were performed in accordance with the guidelines of the Toranomon Hospital Committee on Animal Research.

Biochemical determination of blood and urine

Plasma urea nitrogen, creatinine, calcium, and phosphorus were measured by automated analysis (Model AU-550; Olympus, Tokyo, Japan). Plasma intact parathyroid hormone (PTH) was measured using an intact PTH enzyme-linked immunosorbent assay (ELISA) kit (Immutopics, San Clemente, CA, USA). Plasma vitamin D_3 metabolites were measured by competitive protein binding assay using a vitamin D–deficient serum.

Urinary protein concentration was measured by the pyrogallol red method (Wako Pure Chemical Industries, Ltd., Osaka, Japan). Urinary creatinine concentration measured similarly to the plasma creatinine was used for the calculation of creatinine clearance.

Northern blot analysis

Total RNA from the remnant kidney was prepared by the acid guanidinium thiocyanate-phenol-chloroform procedure using Trizol (Gibco BRL, Life Technologies, Inc., Gaithersburg, MD, USA). Polyadenylated [poly $(A)^+$]-enriched RNA was purified using Oligotex dT 30 (TaKaRa Biomedicals). Five micrograms of poly$(A)^+$ RNA was separated by electrophoresis in denaturing agarose gels (2.2 mol/L formaldehyde and 1.5% agarose), transferred to a nylon membrane by diffusion (Hybond N; Amersham Pharmacia Biotech, Bucks, United Kingdom), and cross-linked by ultraviolet irradiation. Hybridization was performed as described previously [11].

The following cDNA fragments were used as probes: a 1.5 kb Eco RI-Bam HI fragment of rat cDNA encoding CYP27B1 [11], a 1.8 kb Kpn I and Acc I fragment of rat cDNA encoding CYP24 [12], a 0.4 kb Eco RI fragment of rat cDNA encoding megalin [8], a 1.8 kb Eco RI fragment of rat cDNA encoding vitamin D receptor (VDR) [13] and a 0.4 kb Hinf I fragment of human cDNA encoding β-actin.

The membranes were analyzed by a PhosphorImager (Bas 2000; Fuji Film, Tokyo, Japan) and then subjected to autoradiography (X-Omat; Eastman Kodak, Rochester, NY, USA). The same membrane was used for the subsequent hybridizations with other probes after stripping. The expression of β-actin mRNA was used as an internal reference standard according to a previous report [14, 15]. β-actin mRNA expression was observed to be unaffected by nephrectomy when an equal amount of poly$(A)^+$ RNA from either the sham-operated or nephrectomized rats was loaded in each lane during each experiment.

Statistical analysis

All numerical data are expressed as mean $\pm$ SD. Data between two groups were analyzed by the unpaired t test, and data among three groups were analyzed by analysis of variance (ANOVA) followed by Bonferroni's post hoc test (significance was attained where $P < 0.0167$). A P value less than 0.05 was considered significant unless otherwise stated.

RESULTS

Animal data

Body weights of the nephrectomized rats were always lower than those of the sham-operated rats at the respective experimental date as shown in Table 1. Hematocrit of the nephrectomized rats decreased with time compared with that of the control rats, probably due to the progression of renal anemia. The plasma concentrations of urea nitrogen as well as creatinine were stable in

Table 1. Body weight and biochemical parameters in sham-operated and nephrectomized rats at weeks 2, 4, and 8 after nephrectomy

Week	2	4	8
Body weight *g*			
Sham-operated	369 ± 17	434 ± 13[b]	476 ± 17[b,c]
Nephrectomized	324 ± 12[a]	362 ± 32[a]	436 ± 25[a,b,c]
Hematocrit %			
Sham-operated	39 ± 1	40 ± 2	45 ± 3[b,c]
Nephrectomized	39 ± 5	35 ± 2[a]	37 ± 3[a]
Plasma urea nitrogen *mg/dL*			
Sham-operated	21 ± 2	21 ± 2	17 ± 2
Nephrectomized	60 ± 8[a]	61 ± 7[a]	54 ± 10[a]
Plasma creatinine *mg/dL*			
Sham-operated	0.40 ± 0.02	0.43 ± 0.03	0.40 ± 0.01
Nephrectomized	0.93 ± 0.15[a]	0.95 ± 0.11[a]	1.10 ± 0.30[a]
Creatinine clearance *mL/min*			
Sham-operated	2.14 ± 0.33	2.69 ± 0.39	4.04 ± 0.77[b,c]
Nephrectomized	0.89 ± 0.18[a]	0.96 ± 0.18[a]	1.19 ± 0.25[a,b]
Urinary protein *mg/day*			
Sham-operated	6.5 ± 2.8	9.9 ± 5.0	7.2 ± 3.6
Nephrectomized	5.7 ± 2.5	12.6 ± 6.4	33.0 ± 17.5[a,b,c]

Results are expressed as mean ± SD of five rats (sham-operated) and eight rats (nephrectomized).
[a] $P < 0.05$ vs. sham-operated
[b] $P < 0.0167$ vs. week 2
[c] $P < 0.0167$ vs. week 4

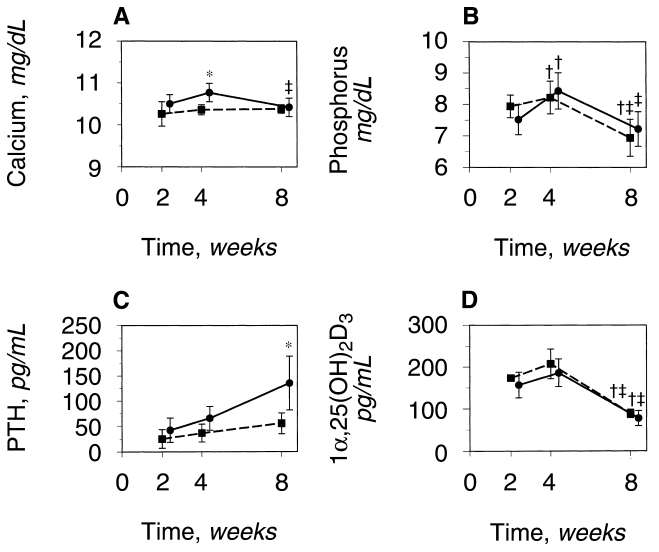

Fig. 1. Time course of plasma concentrations of calcium (*A*), phosphorus (*B*), parathyroid hormone (PTH) (*C*), and 1α,25(OH)$_2$D$_3$ (*D*) in the sham-operated (■) and nephrectomized rats (●). Measurement was performed at weeks 2, 4, and 8 after nephrectomy. Data are mean ± SD of five rats (sham-operated) and eight rats (nephretomized). *$P < 0.05$ vs. sham-operated rats; †$P < 0.0167$ vs. week 2; ‡$P < 0.0167$ vs. week 4.

the sham-operated control, whereas those values in the nephrectomized rats doubled at week 2 and remained constant thereafter. Creatinine clearance in the nephrectomized rats ranged from 30% to 40% of that of the control throughout the experiment. These renal function data are consistent with the moderate degree of renal failure reported by a previous observation [16]. Urinary protein excretion only increased in the nephrectomized rats at week 8.

Table 2. Plasma concentration of vitamin D metabolites at week 8 after nephrectomy

	25(OH)D$_3$ *ng/mL*	1α,25(OH)$_2$D$_3$ *pg/mL*	24,25(OH)$_2$D$_3$ *ng/mL*
Sham-operated	24.8 ± 4.5	70.7 ± 6.8	8.0 ± 0.6
Nephrectomized	39.3 ± 15.0	76.9 ± 10.5	8.6 ± 3.1

Values are expressed as mean ± SD of five (sham-operated) and eight rats (nephrectomized).

Blood chemistry

Timed changes in the plasma concentrations of calcium, phosphorus, intact PTH, and 1α,25(OH)$_2$D$_3$ are shown in Figure 1. Plasma calcium transiently increased at week 4 in the nephrectomized rats, but did not change with time in the sham-operated animals. Plasma phosphorus increased at week 4 and decreased at week 8, but no difference was obtained between the sham-operated and nephrectomized rats.

Intact PTH in the nephrectomized rats appeared to increase with time but was not significant ($P = 0.108$). When compared to the sham-operated rats, intact PTH at week 8 was significantly greater in the nephrectomized rats ($P = 0.040$) as shown in Figure 1C. Plasma 1α,25(OH)$_2$D$_3$ decreased at week 8 in both the sham-operated and nephrectomized rats but no significant difference was achieved between the two (Fig. 1D).

With respect to other vitamin D metabolites, the plasma concentrations of 25(OH)D$_3$ and 24,25(OH)$_2$D$_3$ were measured by competitive protein binding assay (CPBA) using vitamin D–deficient serum (Table 2). In addition to there being no changes in 1α,25(OH)$_2$D$_3$ between the sham-operated and nephrectomized rats, the other two metab-

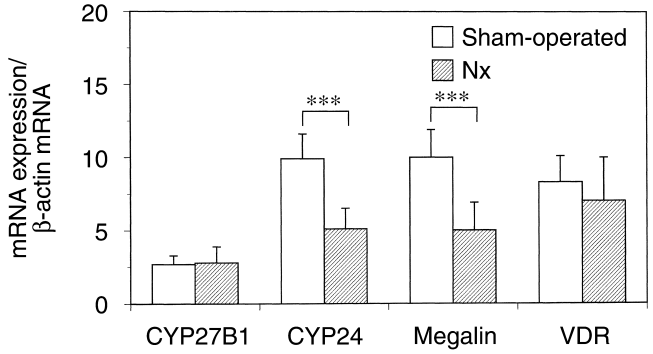

Fig. 2. Northern blot of CYP27B1, CYP24, megalin, and vitamin D receptor (VDR) mRNAs at week 2 in the sham-operated and nephrectomized rats. Messenger RNA expression of the target genes was corrected for respective β-actin mRNA by reprobing. Values are mean ± SD of 7 and 11 rats for sham-operated and 3/4 nephrectomy (Nx), respectively. ***$P < 0.001$.

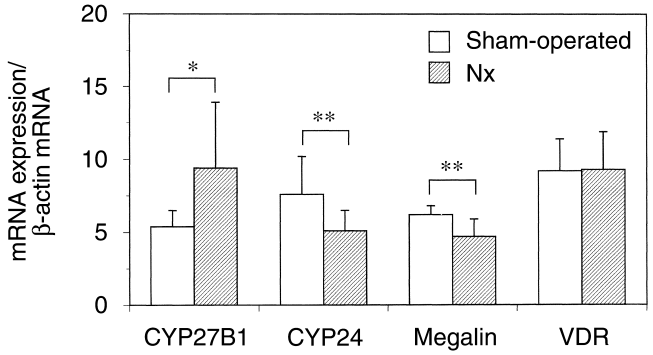

Fig. 3. Northern blot of CYP27B1, CYP24, megalin, and vitamin D receptor (VDR) mRNAs at week 4 in the sham-operated and nephrectomized rats. Messenger RNA expression of the target genes was corrected for respective β-actin mRNA by reprobing. Values are mean ± SD of 7 and 11 rats for sham-operated and 3/4 nephrectomy (Nx), respectively. *$P < 0.05$; **$P < 0.01$.

olites did not vary either. No correlation was found between any two variables among the three metabolites in the sham-operated group or nephrectomized group (data not shown).

Northern blot analysis of CYP27B1, CYP24, megalin, and VDR expression

Next, we performed Northern blot analysis to examine the gene expression of CYP27B1, CYP24, megalin, and VDR in the sham-operated ($N = 7$) and nephrectomized rats ($N = 11$) at weeks 2, 4, and 8 following nephrectomy. The expression of these genes was corrected for β-actin expression of the same blot filter by reprobing. While CYP27B1 or VDR mRNA expression was unaltered, the expression of CYP24 and megalin mRNAs were significantly decreased at week 2, compared with those in the sham-operated rats (Fig. 2). At week 4, CYP27B1 mRNA began increasing in the nephrectomized rats while CYP24 and megalin mRNAs were decreased (Fig. 3). Finally, at week 8, an increase in CYP27B1 mRNA was more evident in the nephrectomized rats and the decreases in both CYP24 and megalin mRNA were still observed (Fig. 4). In addition, VDR mRNA was decreased by half in the nephrectomized rats at week 8 (Fig. 4). A representative result obtained from the sham-operated and nephrectomized rats at week 8 is shown in Figure 5.

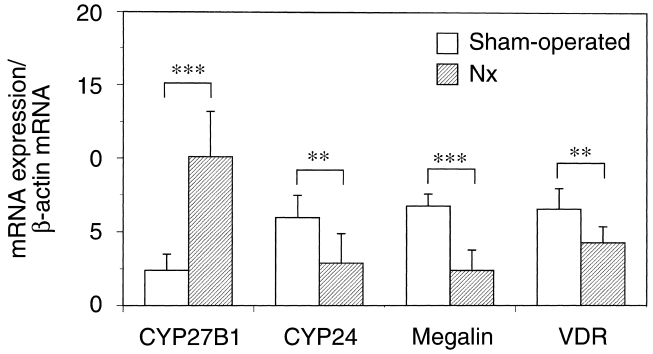

Fig. 4. Northern blot of CYP27B1, CYP24, megalin, and vitamin D receptor (VDR) mRNAs at week 8 in the sham-operated and nephrectomized rats. Messenger RNA expression of the target genes was corrected for respective β-actin mRNA by reprobing. Values are mean ± SD of 7 and 11 rats for sham-operated and 3/4 nephrectomy (Nx), respectively. **$P < 0.01$; ***$P < 0.001$.

DISCUSSION

The 3/4 nephrectomy rat model used in the present study exemplified a moderate degree of renal failure ranging from 30% to 40% of residual renal function. The degree of renal failure is thought to be less severe than that of 5/6 nephrectomy at the same experimental time after nephrectomy [10]. Our renal function data almost corroborated with the previously reported data of moderate renal failure induced by 5/6 nephrectomy for 2 weeks [16]. In fact, the 3/4 nephrectomy rats revealed no significant change in the plasma levels of calcium, phosphorus, or $1\alpha,25(OH)_2D_3$ compared to the sham-operated control rats over 8 weeks (the maximal observation period of the present study). The findings are quite similar to a moderate degree of renal failure in which the serum concentration of $1\alpha,25(OH)_2D_3$ is kept constant as well as calcium and phosphorus [17, 18]. Thus, the 3/4 nephrectomy rat model turned out to be suitable for the present study since we aimed at investigating the timed changes in vitamin D hydroxylase gene expression. Plasma intact PTH at week 8 was found to increase in the nephrectomized rats when compared to that of the sham-operated rats, suggesting that the very early stage of secondary hyperparathyroidism might be-

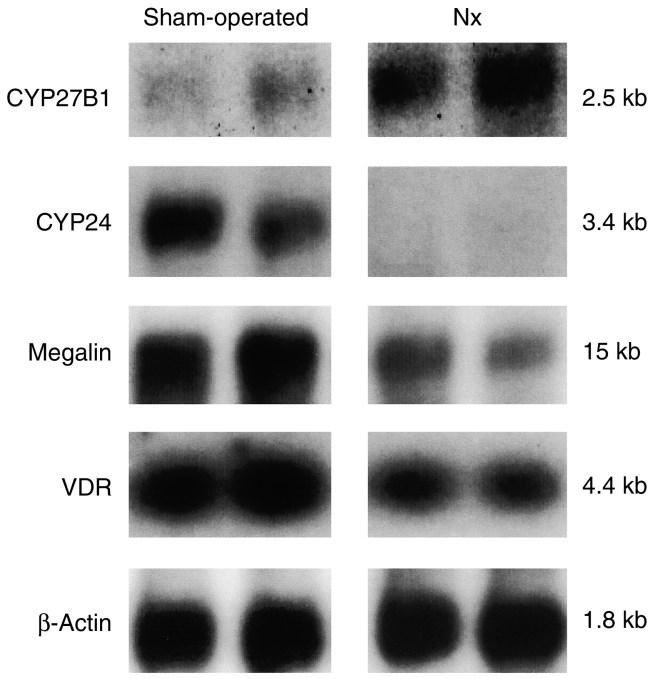

Fig. 5. Northern hybridization of CYP27B1, CYP24, megalin, and vitamin D receptor (VDR) mRNAs from the sham-operated and nephrectomized (Nx) at week 8. Sizes of the respective mRNA are shown to the right.

gin in order to compensate for calcium and vitamin D metabolism.

Using this 3/4 nephrectomy model, we demonstrated that the expression of CYP27B1 mRNA was significantly increased at week 8 in the remnant kidneys, compared with that in the sham-operated rats. Since plasma PTH concentration was elevated at week 8, the observed increase in CYP27B1 mRNA level can be ascribed to PTH-dependent mechanisms, as underscored by the currently prevailing notion [1]. In keeping with this view, Murayama et al [19] found that in a more advanced 5/6 nephrectomy rats, CYP27B1 mRNA expression was poorly responsive to PTH administration, leading to a decrease in CYP27B1 mRNA in the face of a marked increase in plasma PTH. In addition, their study revealed a slight, yet, significant increase in the plasma phosphorus [19]. In this regard, hyperphosphatemia has been known to decrease the enzymatic activity of CYP27B1 as well as the concentration of plasma $1\alpha,25(OH)_2D_3$ [17, 20, 21], while reduction in the plasma phosphorus has been shown to be a stimulator of the plasma $1\alpha,25(OH)_2D_3$ [22, 23] . In fact, we showed previously that the expression of *CYP27B1* gene is moderately stimulated in response to a low phosphorus diet [11]. These observations suggest that in the moderate stage of renal failure with increased PTH and the normal level of plasma phosphorus, *CYP27B1* gene is up-regulated via PTH-dependent mechanisms, contributing to the maintenance of plasma $1\alpha,25(OH)_2D_3$. As loss of functioning nephrons progresses, the remnant kidney advances to a state where PTH responsiveness of renal cells is diminished, allowing a decrease in CYP27B1 mRNA.

In contrast, the expression of *CYP24* gene decreased as early as week 2, well before the elevation of PTH. Several studies have suggested that the renal 24-hydroxylation reaction of $25(OH)D_3$ is reduced in renal failure [5, 16, 24–26]. In accordance with these findings, the expression of renal CYP24 mRNA in the nephrectomized rats was lower than that in the sham-operated rats throughout the experiment, whereas the plasma level of $24,25(OH)_2D_3$ did not differ in the present study. The mechanism of reduced CYP24 mRNA may involve several elements. First, PTH is known to inhibit CYP24 in the kidney [27, 28]. Second, down-regulation of VDR may be involved; VDR mRNA was indeed decreased at week 8 as shown in the present study. Transcription of the *CYP24* gene is regulated by the cis-arrayed vitamin D responsive element upon binding to a complex of VDR, $1\alpha,25(OH)_2D_3$, and retinoid X receptor [29]. However, the fact that both the increase in PTH and the modest decline in VDR mRNA were found only at week 8 in the present study may not fully account for the mechanism. Uremic toxins, for example, could be taken into consideration. Indeed, Hsu, Patel, and Young [26] reported that uremic plasma ultrafiltrate decreases 24-hydroxylase activity, thereby reducing $1\alpha,25(OH)_2D_3$ degradation.

Taken together, it is worth mentioning about the balance between two hydroxylase genes, *CYP27B1* and *CYP24*, in reduced nephron mass. Despite having one-fourth the nephron mass the plasma concentration of $1\alpha,25(OH)_2D_3$ was unchanged in the present study, indicating that an increase in CYP27B1 or a decrease in CYP24 or both may be necessary. Our data suggest that the decrease in CYP24 first occurs and the increase in CYP27B1 comes into play later. The reciprocal regulation of synthesizing and degradative enzymes forms a general scheme of $1\alpha,25(OH)_2D_3$ metabolism [30, 31]. The present study clearly demonstrated the increase in CYP27B1 and the decrease in CYP24 expression in the moderate chronic renal failure. In addition, the increase in CYP27B1 mRNA may be supported by a previous finding in which renal $25(OH)D_3$-1α-hydroxylase after uninephrectomy was unchanged in a unit of animal but increased in a unit of mg DNA before hypertrophy [32]. Theoretically, when one-fourth of the kidney is resected, a fourfold increase in CYP27B1 mRNA is required in order to maintain the plasma $25(OH)D_3$ level. In this regard, the down-regulation of CYP24 may play a more important role in the very early stage of renal failure than CYP27B1.

The recent observation from the megalin knockout experiment has added a new scheme to the conventional

framework of vitamin D metabolism. Nykjaer et al [6] have shown that circulating $25(OH)D_3$ in complex with vitamin D binding protein (DBP) is filtered through the glomerulus and reabsorbed in the proximal tubules by the endocytic receptor megalin. Thus, abnormal urinary excretion of $25(OH)D_3$ and DBP seen in megalin knockout mice results in severe vitamin D deficiency and bone disease [6]. In the present study, we found the expression of megalin mRNA greatly decreased in the remnant kidney of the nephrectomized rats. In this setting, the number of $25(OH)D_3$ molecules taken up via the megalin pathway might be tremendously reduced in the proximal tubular cells because the GFR also declines. Given the unaltered plasma $1\alpha,25(OH)_2D_3$ level, two compensatory mechanisms are conceivable. Most probably, the coordinated changes in both the marked increase in CYP27B1 and decrease in CYP24 could overcome the dramatic decline of megalin expression. Alternatively, some synthesis of $1\alpha,25(OH)_2D_3$ is maintained by nonmegalin-mediated mechanisms; several groups have reported the expression of CYP27B1 in more distal segments of the nephron [33] and at extrarenal sites [34]. Although the decreased expression of megalin has not been reported previously, more studies need to be done to determine why megalin mRNA decreases in the early stage of renal failure and to clarify its pathophysiologic implication.

CONCLUSION

The present study demonstrates that in moderate renal failure CYP27B1 mRNA remarkably increases with a reciprocal alteration of CYP24 mRNA. A dramatic variation of the two key enzymes for vitamin D activation and degradation accounts for an effective compensatory mechanism in the face of reduced nephron mass and down-regulation of megalin mRNA. However, the signal for this reciprocal alteration of the two enzymes remains unclear in the present study, although PTH and VDR may partly contribute to this pathophysiology. Our present findings elucidate the molecular mechanism of the elaborate vitamin D metabolism in moderate renal failure, and thus provide new insights into the understanding and potential treatment of secondary hyperparathyroidism.

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